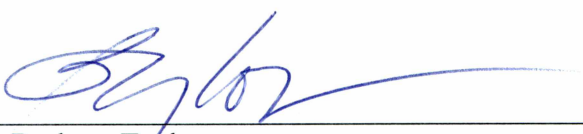


LINKAGES BETWEEN PROTEIN UBIQUITINATION, PROTEASOME ACTIVITY
AND THE EXPRESSION OF OXYGEN-BINDING PROTEINS IN ANTARCTIC
NOTOTHENIOID FISHES

By

Corey A. Oldham

RECOMMENDED:



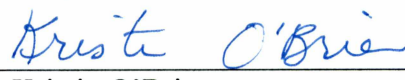
Dr. Barbara Taylor

Advisory Committee Member




Dr. Kriya Dunlap

Advisory Committee Member



Dr. Kristin O'Brien

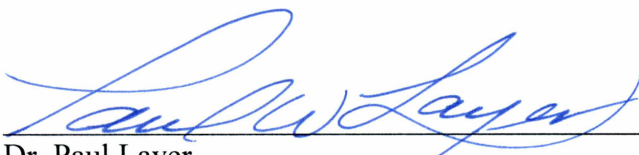
Advisory Committee Chair



Dr. Diane Wagner

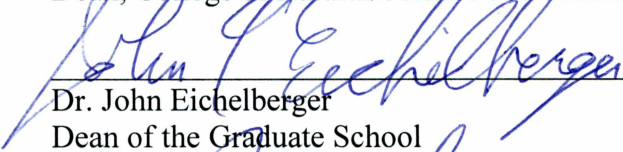
Chair, Department of Biology and Wildlife

APPROVED:



Dr. Paul Layer

Dean, College of Natural Science and Mathematics



Dr. John Eichelberger

Dean of the Graduate School



Date

LINKAGES BETWEEN PROTEIN UBIQUITINATION, PROTEASOME ACTIVITY
AND THE EXPRESSION OF OXYGEN-BINDING PROTEINS IN ANTARCTIC
NOTOTHENIOID FISHES

A
THESIS

Presented to the Faculty
of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

Corey A. Oldham, B.S.

Fairbanks, Alaska

December 2015

Abstract

Antarctic icefishes lack hemoglobin (Hb), and some species lack cardiac myoglobin (Mb). As iron-centered proteins, Hb and Mb can promote the formation of reactive oxygen species that may damage biological macromolecules. Consistent with this, we find higher levels of oxidized proteins in some tissues of red-blooded notothenioids than in icefishes. Oxidized proteins are marked for degradation by the conjugation of the protein ubiquitin. I hypothesized that levels of ubiquitinated proteins and 20S proteasome activity (which degrades oxidized proteins) would be higher in +Hb and +Mb notothenioids than icefishes lacking the proteins. Levels of ubiquitinated proteins and rates of proteasome activity were measured in the heart ventricle, pectoral adductor, and liver of six species of notothenioids differing in Hb and Mb expression. Previous studies in notothenioids suggest that oxidative stress declines following acclimation to 4°C. I also hypothesized that levels of ubiquitinated proteins and 20S proteasome activity would decline in response to acclimation to 4°C. Levels of ubiquitinated proteins and rates of proteasome activity were measured in the heart ventricle, pectoral adductor, and liver of the red-blooded *Notothenia coriiceps* held at ambient temperature and acclimated to 4°C for 3 weeks. Levels of ubiquitinated proteins were higher in tissues of the red-blooded *N. coriiceps* compared to icefishes, but the activity of the 20S proteasome did not follow a similar trend, suggesting that icefishes do not incur an energetic benefit resulting from reduced rates of protein degradation. Levels of ubiquitinated proteins were equivalent in heart ventricle and oxidative skeletal muscle, and proteasome activities were equivalent in all tissues between acclimated *N. coriiceps* and those held at ambient temperature, suggesting that protein damage and rates of protein degradation are not altered in notothenioids by long-term exposure to 4°C.

Table of Contents

Signature Page	i
Title Page	iii
Abstract	v
Table of Contents	vii
List of Figures	ix
Acknowledgments	xi
General Introduction	1
Chapter 1: Linkages Between Protein Ubiquitination, Proteasome Activity and the Expression of Oxygen-Binding Proteins in Antarctic Notothenioid Fishes.....	5
1.1 Summary	5
1.2 Introduction	6
1.3 Material and Methods	9
1.3.1 Fish Collection	9
1.3.2 Levels of Ubiquitinated Proteins	10
1.3.3 20S Proteasome Activity	11
1.3.4 Statistical Analysis	12
1.4 Results	13
1.4.1 Levels of Ubiquitinated Proteins	13
1.4.2 20S Proteasome Activity	13
1.4.3 Effects of Warm Acclimation on Protein Degradation	14
1.5 Discussion	15
1.5.1 Levels of Ubiquitinated Proteins are Higher in Red-blooded Notothenioids than Icefishes	15
1.5.2 Rates of Protein Degradation by the 20S Proteasome Are Not Related to Levels of Ubiquitinated Proteins	16

1.5.3 The Ubiquitin-Proteasome Pathway Displays a Limited Response to Warm Acclimation	17
1.6 Conclusions.....	19
Acknowledgements	19
List of Symbols and Abbreviations.....	19
Funding	20
Author Contributions	20
References.....	21
General Conclusions	31
References.....	33
Appendix.....	37

List of Figures

Figure 1.1 Levels of ubiquitinated proteins in tissues of notothenioids that vary in the expression of hemoglobin (Hb) and myoglobin (Mb)	28
Figure 1.2 Activity of the 20S proteasome in tissues of notothenioids that vary in the expression of hemoglobin (Hb) and myoglobin (Mb)	29
Figure 1.3 Effect of warm acclimation on protein ubiquitination and activity of the 20S proteasome	30

Acknowledgments

I would like to express my deep thanks to my academic advisor Kristin O'Brien for her guidance throughout my graduate studies. Were it not for Kristin's optimism, patience, and good humor I may not have gotten through this process. You will have my lifelong appreciation for bringing me into this project and giving me the opportunity to do my fieldwork at Palmer Station, Antarctica.

I would also like thank my committee members Barb Taylor and Kriya Dunlap for their support. Thanks to all of those I had the good fortune of working with in the O'Brien Lab (Laura, Kelly, Megan, Jessica, Autumn, Luke, Alyssia, Kristen, and Anna) for your friendship and assistance, and for helping to create a wonderful working environment.

I would like to acknowledge our Antarctic collaborators, Lisa, Theresa, Amanda, and Johanne for your friendship and support while on The Ice, as well as the crew aboard the ARV Laurence M. Gould and the Palmer Station support staff, without whose assistance none of this research would have been possible.

Thanks to all my friends and family who have stood by my side (even if only metaphorically) the whole way.

General Introduction

The Southern Ocean surrounding Antarctica is a unique marine environment with 88% of the Antarctic fish fauna endemic to the region (Eastman, 1993). Approximately 22-25 mya the Antarctic Circumpolar Current formed, causing a steep decline in the water temperature of the Southern Ocean and resulting in widespread extinctions in the fish fauna (Kennett, 1977; Clarke and Johnston, 1996). Ancestors of the suborder Notothenioidei, equipped with antifreeze glycopeptides, were among the few survivors and they radiated to fill a wide array of niches. Today, notothenioids are the most diverse and dominant fish taxa in the Southern Ocean, comprising approximately 45% of the 222 benthic fish species native to the Antarctic continental shelf (Eastman, 2005). The relaxed selective pressure brought on by the Oligocene extinctions is thought to have provided adequate conditions for one of the most unique characteristics among Antarctic fishes to evolve, the loss of erythrocytes and oxygen-binding proteins hemoglobin (Hb) and myoglobin (Mb) within the family Channichthyidae.

Antarctic icefishes (suborder Notothenioidei, family Channichthyidae) are the only vertebrates that do not express Hb as adults (Ruud, 1954). Furthermore, six of the 16 species of icefishes lack Mb in their heart ventricle (Sidell et al., 1997; Moylan and Sidell, 2000; Grove et al., 2004). These losses are considered to be neutral mutations (Sidell et al., 1997; Moylan and Sidell, 2000); however, as iron-centered proteins, Hb and Mb may contribute to the production of reactive oxygen species (ROS) that may cause oxidative stress (Tappel, 1953; King et al., 1964). Oxidative stress occurs when the production of ROS exceeds an organism's antioxidant capacity, resulting in damaged biological macromolecules that must be either repaired or degraded, depending on severity of oxidation (Sies, 1997). The misfolding of proteins caused by mild oxidation may be repaired by molecular chaperones; however, proteins subjected to more severe oxidative damage must be degraded by the 20S proteasome or lysosomes (Hawkins, 1991; Wickner et al., 1999; Dröge, 2002). When proteins become oxidized they are marked for degradation by the covalent attachment of the small (8.5 kDa) protein ubiquitin (Shang et al., 1997; Dudek et al., 2005). The proteasome, a proteolytic protein complex, recognizes ubiquitin and degrades damaged proteins into their constituent amino acids, and proteins are replaced through the energetically demanding process of

protein synthesis (Davies, 2001). Therefore, the loss of Hb and Mb may be advantageous for icefishes by reducing ROS production and therefore, reducing rates of protein degradation and synthesis.

Oxidative stress has been implicated in a wide variety of human diseases; for example, most neurodegenerative diseases (e.g. Parkinson's disease and Huntington's disease) are associated with oxidative damage to DNA, proteins, and/or lipids (Hartley et al., 1993; Lee et al., 2001; Honda et al., 2004). Although cellular respiration is the most prominent source of ROS production in organisms, other endogenous sources, such as Hb and Mb, may play a significant role. The oxidation of Hb and Mb takes place *in vivo* when molecular oxygen reacts with heme in the ferrous state (Fe^{2+}), producing the powerful oxidant superoxide (Reeder and Wilson, 2005). Further, hydrogen peroxide can react with heme in the ferric state (Fe^{3+}) to produce two additional oxidants, ferryl iron ($\text{Fe}^{4+}=\text{O}_2^-$) and a free radical located on an amino acid residue of the globin protein (Reeder and Wilson, 2005). Currently, it is unknown to what extent Hb and Mb affect oxidative stress. The hemoglobin-null state and variable expression of cardiac Mb in Antarctic icefishes presents a suitable model with which to study the role of Hb and Mb in oxidative stress.

The Western Antarctic Peninsula is one of the fastest-warming regions in the world (King and Harangozo, 1998; Vaughan et al., 2003; Turner et al., 2005). The surface waters of the Western Antarctic Peninsula have warmed approximately 1°C during the last 50 years, and subsurface waters are projected to increase 0.4 to 0.6°C during the next century, with an increase of as much as 1°C by the year 2200 (Meredith and King, 2005; Yin et al., 2011). It is unknown how notothenioids will fare in a warming climate. What is known, is that notothenioids are intolerant of increased temperatures and extremely stenothermic (Somero and DeVries, 1967) as a result of their evolution in an extremely cold and stable environment. Water temperatures in McMurdo Sound are a nearly constant -1.9°C , while that of the Western Antarctic Peninsula region ranges seasonally from approximately 1.5°C to -1.8°C (Littlepage, 1965; DeWitt and Bushnell, 1971).

The results of some studies suggest that acclimation of notothenioids to 4°C reduces oxidative stress (Bilyk and Cheng, 2014; Enzor and Place, 2014; Mueller et al.,

2014). In addition, cold-adapted Antarctic fishes have higher levels of ubiquitinated proteins compared to temperate fishes, suggesting that cold temperature denatures proteins (Todgham et al., 2007) and that warming may reduce levels of ubiquitinated proteins and rates of protein degradation for notothenioids.

I tested the overarching hypothesis that the loss of Hb and Mb in Antarctic icefishes may be advantageous because it reduces oxidative stress and rates of protein degradation. My specific hypotheses were:

(1). The loss of Hb and Mb reduces oxidative stress, resulting in lower levels of ubiquitinated proteins and rates of protein degradation by the 20S proteasome compared to notothenioids that express the proteins. To test this hypothesis, I measured levels of ubiquitinated proteins and rates of 20S proteasome activity in the heart ventricle, pectoral adductor muscle, and liver of six species of notothenioids differing in the expression of Hb and Mb: *Chaenocephalus aceratus* (-Hb/-Mb), *Champscephalus gunnari* (-Hb/-Mb), *Chionodraco rastrispinosus* (-Hb/+Mb), *Pseudochaenichthys georgianus* (-Hb/+Mb), *Gobionothus gibberifrons* (+Hb/+Mb), and *Notothenia coriiceps* (+Hb/+Mb).

(2). Acclimation of notothenioids to 4°C for 3 weeks will reduce protein denaturation and thus levels of ubiquitinated proteins and activity of the 20S proteasome compared to animals held at ambient temperature. To test this hypothesis, I measured levels of ubiquitinated proteins and rates of 20S proteasome activity in three highly oxidative tissues (heart ventricle, pectoral adductor muscle, and liver) of *N. coriiceps* held at ambient temperature and acclimated to 4°C for 3 weeks.

Chapter 1: Linkages Between Protein Ubiquitination, Proteasome Activity and the Expression of Oxygen-Binding Proteins in Antarctic Notothenioid Fishes¹

1.1 Summary

Antarctic icefishes (suborder Notothenioidei, family Channichthyidae) lack hemoglobin (Hb) and some species lack cardiac myoglobin (Mb). As iron-centered proteins, Hb and Mb can promote the formation of reactive oxygen species that may damage macromolecules. Consistent with this, levels of oxidized proteins are lower in icefishes compared to some red-blooded notothenioids. Additionally, previous studies have suggested that oxidative stress declines in notothenioids in response to acclimation to 4°C. We hypothesized that levels of ubiquitinated proteins and activity of the 20S proteasome, which degrades oxidized proteins, would be lower in icefishes compared to red-blooded fishes, and would decline in response to acclimation to 4°C. Levels of ubiquitinated proteins and rates of proteasome activity were measured in the heart ventricle, pectoral adductor muscle, and liver of six species of notothenioids differing in Hb and Mb expression, and in these same tissues of the red-blooded *Notothenia coriiceps* held at ambient temperature and acclimated to 4°C for 3 weeks. While levels of ubiquitinated proteins were lower in icefishes compared to the red-blooded *N. coriiceps*, the activity of the 20S proteasome was similar among species, suggesting that lack of oxygen-binding proteins does not reduce rates of protein degradation. Levels of ubiquitinated proteins were higher in liver of warm-acclimated *N. coriiceps* compared to ambient animals. However, levels of ubiquitinated proteins were equivalent in heart ventricle and oxidative skeletal muscle, and proteasome activity was equivalent between warm-acclimated and ambient animals in all tissues, suggesting warm acclimation to 4°C does not alter protein damage or degradation rates in *N. coriiceps*.

¹ Oldham, C.A., Fish, A.A. and O'Brien, K.M. Linkages Between Protein Ubiquitination, Proteasome Activity and the Expression of Oxygen-Binding Proteins in Antarctic Notothenioid Fishes. Submitted to *Journal of Experimental Biology*

1.2 Introduction

Approximately 22-25 mya the Southern Ocean became oceanographically isolated by the formation of the Antarctic Circumpolar Current, at which time ocean temperatures also began to precipitously decline, decreasing from approximately 10 to 5°C by the late Miocene (Clarke and Johnston, 1996; Kennett, 1977). The concurrent expansion of ice sheets resulted in habitat loss and changes in the ecosystem trophic structure, leading to extensive extinction of the fish fauna (Clarke and Johnston, 1996). With a variety of new niches available, notothenioids underwent extensive radiation and acquired a wide array of novel traits, including antifreeze glycoproteins, cold-adapted microtubules, and the loss of Hb in icefishes (Albertson et al., 2010; Clarke and Johnston, 1996; Detrich et al., 2000; Eastman, 2005).

Antarctic icefishes (suborder Notothenioidei, family Channichthyidae) are the only vertebrates that lack the oxygen-binding protein hemoglobin (Hb) as adults (Ruud, 1954). The loss of Hb is correlated with a ten-fold reduction in the oxygen-carrying capacity of icefish blood compared to that of Hb-expressing fishes (Hemmingsen and Douglas, 1970; Holeton, 1970). Icefishes have several physiological traits that help to compensate for the lack of Hb, but it is unknown whether these traits arose prior to, or subsequent to, the loss of Hb. For example, icefishes have blood vessels that are two-to-three times the diameter of other notothenioids, and they have two-to-four times the blood volume of red-blooded species (Fitch et al., 1984; Hemmingsen and Douglas, 1970). In addition, icefishes have relatively large hearts (three-to-five times the heart-to-body-mass ratio of red-blooded species) that pump blood at low pressure and high volume, reducing cardiac work (Holeton, 1970). Nevertheless, cardiac work of icefishes is estimated to be 1.2-to-3.4 times higher than that of red-blooded species, and consume as much as 27% of their total energy budget (Hemmingsen et al., 1972; Sidell and O'Brien, 2006). The high metabolic costs associated with cardiovascular remodeling in icefishes suggests that the loss of Hb is a disaptation (a character state that is inferior to the preceding state), or at the very least, a neutral adaptation (Montgomery and Clements, 2000; Sidell and O'Brien, 2006).

In addition to lacking Hb, six of the 16 species of icefishes also lack myoglobin (Mb) in heart ventricle, and all notothenioids lack Mb in oxidative skeletal muscle (Grove

et al., 2004; Moylan and Sidell, 2000; Sidell et al., 1997). Mb is functional at environmental temperatures and enhances cardiac performance when present in notothenioids (Acierno et al., 1997; Cashon et al., 1997). The loss of Mb in the hearts of icefishes is not restricted to a single lineage, but rather has occurred four times during the radiation of the family and by at least three distinct genetic lesions (Sidell et al., 1997; Small et al., 2003; Small et al., 1998), suggesting weak selective pressure for maintaining Mb expression, and that the loss of Mb may be advantageous.

As iron-centered proteins, Hb and Mb can facilitate the production of reactive oxygen species (ROS) that damage biological macromolecules (King et al., 1964; Tappel, 1955). Molecular oxygen reacts with heme in the ferrous state (Fe^{2+}), producing superoxide. Also, heme in the ferric (Fe^{3+}) state reacts with hydrogen peroxide to form two oxidants, ferryl iron ($\text{Fe}^{4+}=\text{O}_2^-$) and a protein-bound free radical on the globin (Reeder and Wilson, 2005). Consistent with this, red-blooded notothenioids have higher activity and transcript levels of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) than icefishes (Cassini et al., 1993; Mueller et al., 2012; Witas et al., 1984). Also, the red-blooded notothenioid *Notothenia coriiceps* has significantly higher levels of protein carbonyls, a form of protein oxidation, in heart ventricle than the icefishes *Chaenocephalus aceratus* and *Chionodraco rastrospinosus* (Mueller et al., 2014; Mueller et al., 2012). Additionally, the red-hearted icefish *C. rastrospinosus* has higher levels of protein carbonyls in heart ventricle than hearts of the myoglobinless *C. aceratus*, suggesting that expression of Mb increases protein oxidation (Mueller et al., 2014; Mueller et al., 2012). Moreover, notothenioids may be particularly susceptible to oxidative damage caused by ROS due to their high capacity for oxidative metabolism (Crockett, 2011; Crockett and Sidell, 1990). They also possess high levels of polyunsaturated fatty acids in their cellular membranes, which enhance membrane fluidity at cold temperatures, but are more highly susceptible to lipid peroxidation than saturated fatty acids (Cosgrove et al., 1987; Crockett, 2011).

Higher levels of oxidized proteins in red-blooded fishes compared to icefishes may warrant higher rates of protein degradation. Oxidized proteins are marked for degradation by the ATP-dependent covalent conjugation of ubiquitin (Dudek et al., 2005; Shang et al., 1997). Ubiquitinated proteins are degraded by the 26S proteasome, another

ATP-dependent process (Davies, 2001; Shringarpure et al., 2003). Oxidized proteins may also be degraded by the 20S proteasome, in an ubiquitin- and ATP-independent process (Davies, 2001; Reinheckel et al., 2000). While both the 26S and 20S proteasome degrade oxidatively-modified proteins (Shang and Taylor, 2011), the 20S proteasome does not require proteins to be ubiquitinated to degrade them, and can therefore degrade oxidatively damaged proteins in cells with compromised ubiquitin conjugation systems (Davies, 2001; Shringarpure et al., 2003). Additionally, the 26S proteasome is more susceptible to oxidative damage than the 20S proteasome, suggesting that the 20S proteasome plays a more prominent role in the degradation of oxidized proteins (Reinheckel et al., 2000).

Degraded proteins must be resynthesized, a process that represents a significant portion of an organism's energy budget. As much as 42% of the oxygen consumption of the Atlantic cod (*Gadus morhua*) is devoted to protein synthesis (Houlihan et al., 1988). Lower levels of oxidized proteins in icefishes may lead to lower levels of ubiquitinated proteins and rates of protein degradation, conferring an energetic benefit to icefishes lacking Hb and Mb.

Studies to date suggest that acclimation of notothenioids to 4°C reduces oxidative stress, which may also reduce rates of protein turnover (Bilyk and Cheng, 2014; Enzor and Place, 2014; Mueller et al., 2014). For example, when exposed to 4°C for 7 days, *C. rastrispinosus* and *N. coriiceps* have reduced transcript levels of the antioxidants SOD and CAT, suggesting reduced oxidative stress (Mueller et al., 2014). Further, while initial exposure to 4°C increases protein carbonyls in gill and liver tissue of *Trematomus bernacchii* and *Pagothenia borchgrevinki*, carbonyl levels decline after 56 days of acclimation compared to animals at ambient temperature (Enzor and Place, 2014). Similarly, levels of oxidized proteins do not increase in the heart ventricle of *N. coriiceps* after 10 days of acclimation to 4°C (Mueller et al., 2012). Additionally, polyubiquitin genes are down regulated in the liver of the notothenioid *P. borchgrevinki* after exposure to 4°C for 4 days (Bilyk and Cheng, 2014).

To determine whether the loss of oxygen-binding proteins in Antarctic icefishes reduces levels of damaged proteins and rates of protein degradation, we measured levels of ubiquitinated proteins and rates of 20S proteasome activity in six species of

notothenioids differing in the expression of Hb and cardiac Mb. We also measured levels of ubiquitinated proteins and the rate of 20S proteasome activity in *N. coriiceps* acclimated to 4°C for 3 weeks and in animals held at ambient temperature to determine whether acclimation to 4°C reduces levels of damaged proteins and rates of protein degradation. We focused our measurements on heart ventricle, oxidative skeletal muscle and liver because these tissues have a high oxidative capacity, and thus, are more prone to oxidative damage.

1.3 Material and Methods

1.3.1 Fish Collection

C. aceratus (1124 ± 306 g) (Lönnerberg 1906), *Champsocephalus gunnari* (717 ± 38 g) (Lönnerberg 1905), *Pseudochaenichthys georgianus* (1528 ± 228 g) (Norman 1937), *C. rastrispinosus* (417 ± 66 g) (DeWitt & Hureau 1979), *Gobionotothen gibberifrons* (772 ± 67 g) (Lönnerberg 1905), and *N. coriiceps* (1898 ± 179 g) (Richardson 1844) were collected from two locations off of the Western Antarctic Peninsula, (Low Island [63° 25' S; 62° 10' W], and Dallmann Bay [64° 10' S; 62° 35' W]), by benthic otter trawl and fish pots using the US ARSV *Laurence M. Gould* during April-June, 2013. Fish were held in recirculating seawater tanks aboard the ship before being transferred to the aquarium at the US Antarctic Research Station, Palmer Station, and held in tanks with recirculating seawater at $0.1 \pm 0.5^\circ\text{C}$. Animals were euthanized with a sharp blow to the head followed by cervical transection. Samples of heart ventricle, pectoral adductor muscle, and liver were harvested, flash frozen in liquid nitrogen, and stored at -80°C .

For warm acclimation, specimens of *N. coriiceps* ($N = 7$) were placed in two 700-L insulated recirculating seawater tanks at $0.1 \pm 0.5^\circ\text{C}$ (3 or 4 fish per tank). Fish were held for 24 h and then the temperature in each tank was increased $0.5^\circ\text{C day}^{-1}$ for six days using 3-KW Electro Titanium inline heaters (Aqualogic, San Diego, CA, USA) plumbed in closed circuit to each tank, until the tanks reached 4°C. Acclimated animals were held at $4 \pm 0.2^\circ\text{C}$ while ambient animals were held in a separate tanks at $0.1 \pm 0.5^\circ\text{C}$ for 22 days, at which time the fish were euthanized as described above and tissues harvested. All methods were approved by the UAF Institutional Animal Care and Use Committee (247598-11).

1.3.2 Levels of Ubiquitinated Proteins

Levels of ubiquitinated protein were measured in the heart, pectoral adductor muscle, and liver (N=6 per species) based on the methods used by Hofmann and Somero (1995) and modified by Todgham et al. (2007). Frozen tissues were finely chopped on an ice-cold stage and then homogenized in five volumes of ice-cold homogenization buffer (4% SDS [w/v], 1 mM EDTA, 50 mM Tris-HCl) supplemented with protease inhibitors [cOmplete Protease Inhibitor Cocktail Tablets, Roche, USA], pH 6.8) using a Tissuemizer homogenizer (Tekmar, Cincinnati, OH, USA). Homogenization was completed using Tenbroeck ground-glass tissue homogenizers. Tissue homogenates were boiled 5 min to denature proteins. Homogenates were then centrifuged at 12,000 g for 15 min at room temperature and the supernatant retained. Protein content of the supernatant was determined using a Bradford protein assay (1976) with bovine serum albumen (BSA) used for the standard curve. Supernatants were stored at -80°C.

Samples were diluted with Tris-buffered saline solution (TBS) (20 mM Tris-HCl, 140 mM NaCl, pH 7.6) to a concentration of 0.5 $\mu\text{g } \mu\text{l}^{-1}$ for ventricle and pectoral adductor muscle samples, and 0.25 $\mu\text{g } \mu\text{l}^{-1}$ for liver. One μl of each sample was pipetted in duplicate or triplicate onto a 12 cm x 10 cm sheet of 0.2 μm nitrocellulose membrane (Amersham, GE Healthcare Life Sciences, Little Chalfont, UK). This amount of protein was determined to be within the linear range of detection and the level of ubiquitination was proportional to the amount of protein spotted on the membrane. The protein was heat-fixed to the membrane at 65°C for 20 min. The membrane was then blocked with 5% nonfat milk powder dissolved in Tween-20 Tris-buffered saline solution (TTBS) (20 mM Tris-HCl, 140 mM NaCl, 0.01% Tween-20, pH 7.6, room temperature) for 1 h. After blocking, the membranes were rinsed twice briefly with TTBS and then 3 times for 5 min each with TTBS. The membranes were incubated at 4°C with the ubiquitin conjugate primary antibody (mono- and polyubiquitinated conjugates mAb produced in mice, Enzo Life Sciences, BML-PW8810, Farmdale, New York, USA), diluted 1:5000 in 5% nonfat milk powder dissolved in TTBS. Incubation times were 12.5 h for ventricle, 15 h for pectoral adductor, and 2 h for liver. The membranes were then rinsed briefly twice with TTBS and then rinsed 3 times for 5 min each with TTBS. The membranes were incubated at room temperature with the secondary antibody (rabbit anti-Mouse IgG

peroxidase antibody, A9044, Sigma-Aldrich, St. Louis, MO, USA), diluted 1:10,000 in 5% nonfat dry milk powder in TTBS. Membranes with ventricle samples were incubated 1.5 h, pectoral adductor muscle samples for 2 h, and liver samples for 2.25 h. The membranes were then rinsed briefly twice with TTBS, washed 3 times for 5 min each in TTBS, and developed using a chemiluminescence kit (Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare), according to manufacturer's specifications. Chemiluminescence was detected for 15 min using AlphaImager 3300 Imaging System (Protein Simple, San Jose, CA, USA) and intensity was quantified using ImageQuant TL software (GE Healthcare). Levels of ubiquitinated proteins were normalized to levels in heart ventricles of *N. coriiceps* (N=6). Samples from each individual were spotted in triplicate on membranes for measurements in heart ventricle and in duplicate for measurements in pectoral adductor muscle and liver. The average value for *N. coriiceps* ventricle (also spotted in triplicate for heart and duplicate for pectoral adductor and liver) was used to normalize values for other species and tissues. Samples from acclimated and ambient animals of *N. coriiceps* were all loaded on the same membrane and so absolute intensities were used to determine levels of ubiquitinated proteins.

1.3.3 20S Proteasome Activity

Activity of the 20S proteasome was measured in the heart, pectoral adductor muscle, and liver (N=8 for each species except for *C. aceratus* ventricle and pectoral adductor muscle for which N=7) based on the method developed by Shibatani and Ward (1995) and adapted for use on fish by Dobly et al. (2004). Frozen tissues were finely chopped on an ice-cold stage and then homogenized in five volumes of ice-cold lysis buffer (50 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1.0 mM β -mercaptoethanol) using a Tissuemizer homogenizer (Tekmar) and Tenbroeck ground-glass tissue homogenizers. The homogenate was centrifuged at 20,000 g for 1 h at 4°C and the supernatant retained. Protein content of the supernatant was determined using a Bradford protein assay (1976) with BSA used for the standard curve.

Proteasome activity was measured using the proteasome-specific fluorogenic substrate LLVY-AMC (Enzo). The substrate was dissolved in DMSO (5.71 mM), then aliquoted and stored at -80°C until use. Although maximal activity of the 20S proteasome

has been shown to require SDS in mammals (Shibatani and Ward, 1995), we tested 20S proteasome activity in notothenioid fishes in a range of SDS concentrations (0% to 0.05%) and it was maximal in Tris buffer lacking SDS, so we omitted it from the reaction mixture. Activity was measured by incubating 50 μg protein from the supernatant with 40 μM LLVY-AMC in 22.5 μl 100 mM Tris-HCl (pH 8.0) for 60 min at 5°C. The reaction was determined to be linear for 90 min. The reaction was stopped by adding 225 μl 0.1 M sodium borate (pH 9.1) and 65 μl 1% SDS. Fluorescence of AMC was determined at excitation/emission wavelengths of 380 and 460nm, respectively, on a Gemini EM Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Parallel samples were prepared by adding the proteasome inhibitor MG-132 (133 μM) (Enzo) prior to incubation. Activity was calculated by determining the concentration of AMC in the samples using the standard curve minus activity in the presence of MG-132. Activity is expressed as $\text{pmol AMC h}^{-1} 50 \mu\text{g}^{-1} \text{ protein}$. The standard curve was prepared using 8 LLVY-AMC concentrations between 3.3 μM and 44 μM . The samples and standard curve were measured in triplicate.

1.3.4 Statistical Analysis

Outliers were identified using box-and-whisker plots created with JMP 7 (SAS Institute, Cary, NC, USA), and were removed from analysis. Significant differences in levels of ubiquitinated proteins and the activity of the 20S proteasome in pectoral adductor muscle among species were determined using a one-way ANOVA followed by a Tukey's post-hoc test using SigmaPlot 11.0 (Systat Software, San Jose, CA, USA). Equal variance was confirmed and normality tested using a Shapiro-Wilk test and the software Sigma Plot 11.0 (Systat Software). A Kruskal-Wallis one-way ANOVA on ranked data using the software Sigma Plot 11.0 (Systat Software) was used to determine significant differences in activity of the 20S proteasome and in levels of ubiquitinated proteins in heart ventricles and liver tissue among species because these data were either not normally distributed and/or lacked equal variance. Pairwise comparisons were made using a Tukey-Kramer Honestly Significant Difference test for the data set that met parametric assumptions with JMP 12 (SAS Institute). Pairwise comparisons were made using a Mann-Whitney Wilcoxon test with a Bonferroni correction ($\alpha = 0.0083$) for data

sets that did not meet parametric assumptions using JMP 12 (SAS Institute). Significant differences in levels of ubiquitinated proteins and activity of the 20S proteasome between acclimated and control specimens were determined using a Student's *t*-test using JMP 7 (SAS Institute).

1.4 Results

1.4.1 Levels of Ubiquitinated Proteins

Levels of ubiquitinated proteins were higher in the heart ventricle of the red-blooded fishes *N. coriiceps* and *G. gibberifrons* compared to icefishes, and levels were higher in the hearts of *N. coriiceps* than in *G. gibberifrons* (Fig. 1.1A, $P < 0.0083$). Ubiquitinated protein levels were 5.2- to 9.5-fold higher in the heart ventricle of *N. coriiceps* than in icefishes, 2.5- to 4.4-fold higher in the heart ventricle of *G. gibberifrons* than icefishes, and 2.1-fold higher in the heart ventricle of *N. coriiceps* than *G. gibberifrons*. Icefishes that express Mb did not have higher levels of ubiquitinated proteins than those that do not.

Levels of ubiquitinated proteins were highest in the pectoral adductor muscle of *N. coriiceps* and did not differ among other species (Fig 1.1B, $P < 0.05$). Ubiquitinated protein levels were 1.6- to 2.4-fold higher in the pectoral adductor muscle of *N. coriiceps* than in other notothenioids.

Levels of ubiquitinated proteins were higher in the liver of the two red-blooded species compared to the icefishes *P. georgianus* and *C. rastrispinosus* (Fig 1.1C, $P < 0.0083$). Ubiquitinated protein levels were 3.6- to 4.6-fold higher in the liver of *N. coriiceps* than in *P. georgianus* and *C. rastrispinosus*, and 2.9- to 3.7-fold higher in the liver of *G. gibberifrons* than in the icefishes *P. georgianus*, and *C. rastrispinosus*.

1.4.2 20S Proteasome Activity

The chymotrypsin-like activity of the 20S proteasome was highest in the heart ventricle of *G. gibberifrons* (483 ± 31 pmol AMC h⁻¹ 50 µg⁻¹ protein) and *N. coriiceps* (438 ± 8 pmol AMC h⁻¹ 50 µg⁻¹ protein), intermediate in icefishes expressing Mb (347 ± 15 in *P. georgianus* and 340 ± 26 pmol AMC h⁻¹ 50 µg⁻¹ in *C. rastrispinosus*), and lowest in the white-hearted icefish *C. aceratus* (280 ± 10 pmol AMC h⁻¹ 50 µg⁻¹ protein)

(Fig. 1.2A, $P < 0.0083$). The exception to this pattern was in the icefish *C. gunnari* (524 ± 59 pmol AMC h⁻¹ 50 µg⁻¹ protein), whose activity was comparable to the red-blooded species (Fig. 1.2A, $P < 0.0083$). 20S proteasome activity in the heart ventricle of the Mb-expressing icefish *P. georgianus* was higher than that of the myoglobinless *C. aceratus* (Fig. 1.2A, $P < 0.0083$), however, activity in the ventricle of the Mb-expressing *C. rastroripinosus*.

There was no difference in 20S proteasome activity in the pectoral adductor muscle among *G. gibberifrons* (246 ± 25 pmol AMC h⁻¹ 50 µg⁻¹ protein) and icefishes (mean = 347 ± 20 pmol AMC h⁻¹ 50 µg⁻¹ protein) (Fig. 1.2B, $P < 0.0083$). Activity was higher in the pectoral adductor muscle of the icefish *C. aceratus* compared to the red-blooded *N. coriiceps* (354 ± 38 pmol AMC h⁻¹ 50 µg⁻¹ protein) (Fig. 1.2B, $P < 0.0083$).

In the liver, 20S proteasome activity was highest in *C. gunnari* (630 ± 80 pmol AMC h⁻¹ 50 µg⁻¹ protein) and was 1.7- to 2.9-fold higher than in the other species (Fig. 1.2C, $P < 0.0083$). 20S proteasome activity was not higher in the liver of red-blooded notothenioids than in the icefishes *C. aceratus*, *P. georgianus*, and *C. rastroripinosus* (Fig. 1.2C, $P > 0.0083$).

1.4.3 Effects of Warm Acclimation on Protein Degradation

Levels of ubiquitinated proteins did not change in response to warm acclimation in the heart ventricle and pectoral adductor muscle of *N. coriiceps* but relative levels of ubiquitinated proteins were 1.9-fold higher in the liver of *N. coriiceps* acclimated to 4°C than in animals at ambient temperature (Fig. 1.3A, $P < 0.05$).

Activity of the 20S proteasome did not change in response to warm acclimation in *N. coriiceps*. There was no significant difference between 20S proteasome activity in acclimated and ambient fishes in heart ventricle (292 ± 53 and 350 ± 26 pmol AMC h⁻¹ 50 µg⁻¹ protein, respectively), pectoral adductor muscle (237 ± 8 and 261 ± 29 pmol AMC h⁻¹ 50 µg⁻¹ protein, respectively), or liver (378 ± 55 and 418 ± 44 pmol AMC h⁻¹ 50 µg⁻¹ protein, respectively) (Fig. 1.3B, $P < 0.05$).

1.5 Discussion

This work represents the first combined measurements of protein ubiquitination and rates of protein degradation in Antarctic notothenioid fishes, and is the first to compare these measurements between red- white-blooded species to assess the impact of protein damage by iron-centered oxygen-binding proteins. Previous studies of protein ubiquitination and rates of protein turnover in notothenioids have focused on transcript abundance, and have been restricted to red-blooded species (Bilyk and Cheng, 2013; Bilyk and Cheng, 2014; Windisch et al., 2014). Our results indicate that although levels of ubiquitinated proteins are higher in notothenioids expressing Hb compared to hemoglobinless icefishes, rates of protein degradation by the 20S proteasome are not, suggesting there is no energetic benefit to the loss of oxygen-binding proteins.

1.5.1 Levels of Ubiquitinated Proteins are Higher in Red-Blooded Notothenioids than Icefishes

While levels of Hb appear to elevate levels of ubiquitinated proteins, Mb does not. In general, red-blooded notothenioids have higher levels of ubiquitinated proteins than icefishes, suggesting Hb increases protein damage. However, there were no significant differences in levels of ubiquitinated proteins between heart ventricles of icefishes expressing Mb compared to those that lack the protein, suggesting that Mb alone does not significantly promote protein ubiquitination. Ubiquitinated proteins are 2.1-fold higher in the heart ventricle of *N. coriiceps* than *G. gibberifrons*, and this corresponds with 1.9-fold higher levels of Hb in *N. coriiceps* (Beers et al., 2010). The red-blooded notothenioid *N. coriiceps* also has 1.6- to 2.4-fold higher levels of ubiquitinated proteins in pectoral adductor muscle than other notothenioids, and ubiquitinated proteins in the liver of *N. coriiceps* are 2.0- to 4.6-fold higher than those of icefishes. The higher levels of ubiquitinated proteins in red-blooded species compared to icefishes are consistent with their higher levels of oxidized proteins. Levels of carbonylated proteins are 3- to 81-fold higher in the heart ventricle of *N. coriiceps* compared to the icefishes *C. rastrispinosus* and *C. aceratus*, respectively (Mueller et al., 2012). Previous studies have also found that increased levels of ubiquitinated proteins are associated with mild oxidative stress (Dudek et al., 2005; Shang et al., 2001; Shang and

Taylor, 2011). For example, brief exposure of lens epithelial cells to the oxidant H₂O₂ induces a transient increase in levels of ubiquitinated proteins as well as increased ubiquitin-conjugation activity (Shang et al., 1997).

1.5.2 Rates of Protein Degradation by the 20S Proteasome Are Not Related to Levels of Ubiquitinated Proteins

Despite the higher levels of ubiquitinated proteins in red-blooded notothenioids compared to icefishes, there was no clear trend in 20S proteasome activity with regard to expression of Hb and Mb. For example, 20S proteasome activity was equivalent in the heart ventricle of *N. coriiceps* to that of the icefishes *C. gunnari*, *P. georgianus*, and *C. rastrispinosus* but lower in the pectoral adductor muscle of *N. coriiceps* compared to all other species. In liver, 20S proteasome activity in the icefish *C. gunnari* was 1.7- to 2.9-fold higher than other notothenioids, in which proteasome activity was equivalent. Together, these data suggest that although the red-blooded notothenioids have higher levels of ubiquitinated and oxidized proteins, they may not degrade proteins at a higher rate.

Pathways other than the 20S proteasome may contribute to the removal of oxidized and ubiquitinated proteins, and may be employed preferentially by notothenioids. For example, molecular chaperones may repair the unfolding and aggregation of moderately oxidized proteins (Hawkins, 1991; Wickner et al., 1999). Chaperones, such as heat shock protein 70 (Hsp70) and Hsp90, recognize and repair damaged or denatured proteins, as well as non-native protein aggregates (Feder and Hofmann, 1999). It has been suggested that cold temperature denatures proteins (Todgham et al., 2007) and consistent with this, red-blooded notothenioids from McMurdo Sound have higher constitutive levels of Hsp/Hsc70 than temperate notothenioids (Buckley et al., 2004; Hofmann et al., 2000; Place and Hofmann, 2005; Place et al., 2004). This may be an adaptation to life at cold body temperatures, and perhaps also to high levels of protein oxidation in red-blooded fishes.

Additionally, a novel isoform of acylpeptide hydrolase (APEH-2) is stable at low temperatures (when compared to APEH-1), and has the ability to efficiently hydrolyze oxidized BSA (Gogliettino et al., 2014). Moreover, the relative expression and activity of

APEH-2 are higher in erythrocytes of the red-blooded notothenioid *T. bernacchii* compared to erythrocyte-like cells of the icefish *Chionodraco hamatus*, suggesting that rates of protein degradation may, in fact, be higher in red-blooded notothenioids (Riccio et al., 2015).

Our results suggest that red-blooded notothenioids do not degrade oxidized proteins and re-synthesize them, but rather persist with higher levels of oxidatively modified proteins compared to icefishes. This is consistent with the results of a recent study indicating that red-blooded notothenioids do not have higher rates of protein synthesis than icefishes, nor is there a significant difference in the energetic cost of protein synthesis in cardiomyocytes and hepatocytes of notothenioids differing in the expression of Hb and/or Mb (Lewis et al., 2015). Although the oxidation of proteins may reflect pathology in some cases, the oxidation of proteins can also play pivotal roles in the regulation of protein function and play a role in cellular signaling. For example, protein kinase C (PKC) is susceptible to oxidative modification, and oxidation stimulates activity of PKC in cells, promoting cell growth (Gopalakrishna and Jaken, 2000). In the future, identification of oxidatively-modified proteins should shed light on the physiological consequences of higher levels of oxidized proteins in red-blooded notothenioids.

1.5.3 The Ubiquitin-Proteasome Pathway Displays a Limited Response to Warm Acclimation

Levels of ubiquitinated proteins in the heart ventricle and pectoral adductor of *N. coriiceps* did not change in response to acclimation to 4°C for 3 weeks. There was, however, a 1.9-fold increase in levels of ubiquitinated proteins in the liver of *N. coriiceps* acclimated to 4°C. In contrast, 20S proteasome activity did not change in response to a 4°C increase in temperature in any tissue measured.

The limited response of the ubiquitin-proteasome pathway (UPP) to warm acclimation suggests an inability to adjust to a 4°C increase in temperature within a timeframe of several (3) weeks. Alternatively, a 4°C increase in temperature may be insufficient to elicit a change in the UPP. Consistent with this, genes that encode some subunits of the proteasome (such as proteasome subunit beta type-7) are highest in the

liver of the Antarctic eelpout (*Pachycara brachycephalum*) at -1° to 0°C, and remain at basal levels at 3° to 5°C, but are down regulated when acclimated to temperatures of 7°C and higher (Windisch et al., 2014). Although they are highly stenothermic, notothenioids can acclimate to increased temperature. For example, when acclimated to 4°C for 4 to 5 weeks, some species of notothenioids can adjust cardiac output, swimming performance, and/or oxygen consumption rates (Franklin et al., 2007; Robinson and Davison, 2008; Seebacher et al., 2005). Notothenioids can also increase thermal tolerance, as measured by the critical thermal maxima, after exposure to 4°C for 1 to 8 weeks (Bilyk and Devries, 2011; Podrabsky and Somero, 2006). It should be noted however, that the majority of these studies were conducted on fish species collected from McMurdo Sound where temperatures are a nearly constant -1.9°C. Specimens of *N. coriiceps* used in this study were collected from the Western Antarctic Peninsula where temperatures average approximately 0°C and change seasonally between -1.5°C in winter and 1.8°C in summer. Acclimation to 4°C in Western Antarctic Peninsula fishes therefore, represents a 4°C increase in temperature, whereas in fishes from McMurdo Sound, this represents ~ 6°C increase in temperature. An increase in water temperature to 4°C may not be great enough to warrant alterations in the UPP. It is also possible that because notothenioids in the Western Antarctic Peninsula experience greater seasonal temperature fluctuations than those in McMurdo Sound, they may be less sensitive to temperature increases.

The duration of acclimation may also be an important factor in the capacity of *N. coriiceps* to acclimate. Prior studies have shown that polyubiquitin genes are down-regulated in the liver of the Antarctic notothenioid *Pagothenia borchgrevinki* after a 4-day exposure to 4°C (Bilyk and Cheng, 2014). However, levels of carbonylated proteins in the livers of 2 species of Antarctic notothenioids (*Trematomus bernacchii*, and *P. borchgrevinki*) increase after a 7-day exposure to 4°C, but then decrease to basal levels or lower after a 56-day acclimation to 4°C (Enzor and Place, 2014). Further, resting metabolic rate increases in *T. bernacchii* during acute exposure to 4°C, but complete metabolic compensation to 4°C occurs after 9 weeks (Sandersfeld et al., 2015). These data indicate that notothenioids may require 4-to-9 weeks to acclimate to warmer water (Podrabsky and Somero, 2006; Sandersfeld et al., 2015). *N. coriiceps* may require a similar amount of time to fully acclimate with regard to the UPP.

1.6 Conclusions

The loss of Hb and Mb does not alter rates of protein degradation by the 20S proteasome and thus loss of these proteins does not likely confer an energetic benefit to icefishes. This supports the hypothesis that the loss of Hb and Mb is a neutral mutation (Sidell and O'Brien, 2006; Sidell et al., 1997). Recent studies show that the lack of cardiac Mb is not a trait unique to channichthyid icefishes. Fishes from a diverse array of habitats, lifestyles, and phylogenies lack cardiac Mb (Macqueen and Johnston, 2014), raising questions regarding the selective pressure for Mb expression. To date, all temperate and tropical fishes that lack cardiac Mb are relatively small, suggesting oxygen diffusion in cardiac tissue lacking Mb may be allometrically constrained (Macqueen and Johnston, 2014). Clearly this does not apply to the large hearts of Antarctic icefishes. Further research will be required to determine the circumstances under which the selective pressure for cardiac Mb expression is relaxed.

The Western Antarctic Peninsula is one of the three fastest-warming regions in the world, with an increase in sea surface temperature of over 1°C over the last 50 years (King and Harangozo, 1998; Meredith and King, 2005; Turner et al., 2005; Vaughan et al., 2003). At this time, the capacity of notothenioids to adjust to a warming environment remains equivocal. Future studies that align acclimation temperatures and duration, and compare acclimation capacity of notothenioids from varying habitat temperatures will help to elucidate this important question.

Acknowledgements

We thank the Masters and crew of the ARSV *Laurence M. Gould* as well as the support staff at the U.S. Antarctic Research Station, Palmer Station, for their assistance in the field.

List of Symbols and Abbreviations

AMC	7-amino-4-methylcoumarin
APEH	acylpeptide hydrolase
ATP	adenosine triphosphate
CAT	catalase

Hb	hemoglobin
Mb	myoglobin
PKC	protein kinase C
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
LLVY-AMC	Succinate-Leucine-Leucine-Valine-Tyrosine-7-amino-4-methylcoumarin
UPP	ubiquitin-proteasome pathway

Funding

This research was funded by a grant from the National Science Foundation [ANT-1043781 to K.OB.]. C.O. and A.F. were supported in part by a University of Alaska Fairbanks (UAF) Biomedical Learning and Student Teaching grant from NIH.

Author Contributions

K.OB. conceived and designed the experiments, K.OB. and C.O. collected experimental animals and performed animal husbandry. C.O. and A.F. performed experiments, analyzed data, and prepared the manuscript and figures. K.OB. and C.O. edited the manuscript.

References

- Acierno, R., Agnisola, C., Tota, B. and Sidell, B.** (1997). Myoglobin enhances cardiac performance in Antarctic icefish species that express the protein. *Am. J. Physiol-Reg. I* **273**, R100-R106.
- Albertson, R. C., Yan, Y. L., Titus, T. A., Pisano, E., Vacchi, M., Yelick, P. C., Detrich, H. W., 3rd and Postlethwait, J. H.** (2010). Molecular pedomorphism underlies craniofacial skeletal evolution in Antarctic notothenioid fishes. *BMC Evol. Biol.* **10**, 4.
- Beers, J. M., Borley, K. A. and Sidell, B. D.** (2010). Relationship among circulating hemoglobin, nitric oxide synthase activities and angiogenic poise in red- and white-blooded Antarctic notothenioid fishes. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **156**, 422-429.
- Bilyk, K. T. and Devries, A. L.** (2011). Heat tolerance and its plasticity in Antarctic fishes. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **158**, 382-390.
- Bilyk, K. T. and Cheng, C. H.** (2013). Model of gene expression in extreme cold - reference transcriptome for the high-Antarctic cryopelagic notothenioid fish *Pagothenia borchgrevinki*. *BMC Genomics* **14**, 634.
- Bilyk, K. T. and Cheng, C. H. C.** (2014). RNA-seq analyses of cellular responses to elevated body temperature in the high Antarctic cryopelagic notothenioid fish *Pagothenia borchgrevinki*. *Mar. Genomics* **18, Part B**, 163-171.
- Bradford, M. M.** (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.
- Buckley, B. A., Place, S. P. and Hofmann, G. E.** (2004). Regulation of heat shock genes in isolated hepatocytes from an Antarctic fish, *Trematomus bernacchii*. *J. Exp. Biol.* **207**, 3649-3656.
- Cashon, R. E., Vayda, M. E. and Sidell, B. D.** (1997). Kinetic characterization of myoglobins from vertebrates with vastly different body temperatures. *Comp. Biochem. Physiol. B Biochem. Mol. Biol* **117**, 613-620.
- Cassini, A., Favero, M. and Albergoni, V.** (1993). Comparative studies of antioxidant enzymes in red-blooded and white-blooded antarctic teleost fish. *Pagothenia*

- bernacchii* and *Chionodraco hamatus*. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* **106**, 333-336.
- Clarke, A. and Johnston, I. A.** (1996). Evolution and adaptive radiation of Antarctic fishes. *Trends Ecol. Evol.* **11**, 212-218.
- Cosgrove, J. P., Church, D. F. and Pryor, W. A.** (1987). The kinetics of the autoxidation of polyunsaturated fatty acids. *Lipids* **22**, 299-304.
- Crockett, E. L.** (2011). Antioxidant potential is positively correlated with mitochondrial enzyme activity in Antarctic and non-Antarctic notothenioid fishes. *Polar Biol.* **34**, 113-118.
- Crockett, E. L. and Sidell, B. D.** (1990). Some pathways of energy metabolism are cold adapted in Antarctic fishes. *Physiol. Zool.* **63**, 472-488.
- Davies, K. J.** (2001). Degradation of oxidized proteins by the 20S proteasome. *Biochimie* **83**, 301-310.
- Detrich, H. W., Parker, S. K., Williams, R. C., Nogales, E. and Downing, K. H.** (2000). Cold adaptation of microtubule assembly and dynamics structural interpretation of primary sequence changes present in the α - and β -tubulins of antarctic fishes. *J. Biol. Chem.* **275**, 37038-37047.
- Dobly, A., Martin, S. A., Blaney, S. C. and Houlihan, D. F.** (2004). Protein growth rate in rainbow trout (*Oncorhynchus mykiss*) is negatively correlated to liver 20S proteasome activity. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **137**, 75-85.
- Dudek, E., Shang, F., Valverde, P., Liu, Q., Hobbs, M. and Taylor, A.** (2005). Selectivity of the ubiquitin pathway for oxidatively modified proteins: relevance to protein precipitation diseases. *FASEB J.* **19**, 1707-1709.
- Eastman, J.** (2005). The nature of the diversity of Antarctic fishes. *Polar Biol.* **28**, 93-107.
- Enzor, L. A. and Place, S. P.** (2014). Is warmer better? Decreased oxidative damage in notothenioid fish after long-term acclimation to multiple stressors. *J. Exp. Biol.* **217**, 3301-3310.
- Feder, M. E. and Hofmann, G. E.** (1999). Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and Ecological Physiology. *Annu. Rev. Physiol.* **61**, 243-282.

- Fitch, N., Johnson, I. and Wood, R.** (1984). Skeletal muscle capillary supply in a fish that lacks respiratory pigments. *Respir. Physiol.* **57**, 201-211.
- Franklin, C. E., Davison, W. and Seebacher, F.** (2007). Antarctic fish can compensate for rising temperatures: thermal acclimation of cardiac performance in *Pagothenia borchgrevinkii*. *J. Exp. Biol.* **210**, 3068-3074.
- Gogliettino, M., Riccio, A., Balestrieri, M., Cocca, E., Facchiano, A., D'Arco, T. M., Tesoro, C., Rossi, M. and Palmieri, G.** (2014). A novel class of bifunctional acylpeptide hydrolases–potential role in the antioxidant defense systems of the Antarctic fish *Trematomus bernacchii*. *FEBS J.* **281**, 401-415.
- Gopalakrishna, R. and Jaken, S.** (2000). Protein kinase C signaling and oxidative stress. *Free Radic. Biol. Med.* **28**, 1349-1361.
- Grove, T. J., Hendrickson, J. W. and Sidell, B. D.** (2004). Two species of Antarctic icefishes (genus *Champsocephalus*) share a common genetic lesion leading to the loss of myoglobin expression. *Polar Biol.* **27**, 579-585.
- Hawkins, A. J. S.** (1991). Protein Turnover: A Functional Appraisal. *Funct. Ecol.* **5**, 222-233.
- Hemmingsen, E. A. and Douglas, E. L.** (1970). Respiratory characteristics of the hemoglobin-free fish *Chaenocephalus aceratus*. *Comp. Biochem. Physiol.* **33**, 733-744.
- Hemmingsen, E. A., Douglas, E. L. and Johansen, K.** (1972). Aortic blood flow and cardiac output in the hemoglobin-free fish *Chaenocephalus aceratus*. *Comp. Biochem. Physiol. A* **43A**, 1045-1051.
- Hofmann, G. E. and Somero, G. N.** (1995). Evidence for protein damage at environmental temperatures: seasonal changes in levels of ubiquitin conjugates and hsp70 in the intertidal mussel *Mytilus trossulus*. *J. Exp. Biol.* **198**, 1509-1518.
- Hofmann, G. E., Buckley, B. A., Airaksinen, S., Keen, J. E. and Somero, G. N.** (2000). Heat-shock protein expression is absent in the Antarctic fish *Trematomus bernacchii* (family Nototheniidae). *J. Exp. Biol.* **203**, 2331-2339.
- Holeton, G. F.** (1970). Oxygen uptake and circulation by a hemoglobinless Antarctic fish (*Chaenocephalus aceratus* Lonnberg) compared with three red-blooded Antarctic fish. *Comp. Biochem. Physiol. A* **34**, 457-471.

- Houlihan, D., Hall, S., Gray, C. and Noble, B.** (1988). Growth rates and protein turnover in Atlantic cod, *Gadus morhua*. *Can. J. Fish. Aquat. Sci.* **45**, 951-964.
- Kennett, J. P.** (1977). Cenozoic evolution of Antarctic glaciation, the circum-Antarctic Ocean, and their impact on global paleoceanography. *J. Geophys. Res.* **82**, 3843-3860.
- King, J. and Harangozo, S.** (1998). Climate change in the western Antarctic Peninsula since 1945: observations and possible causes. *Ann. Glaciol.* **27**, 571-575.
- King, N. K., Looney, F. D. and Winfield, M. E.** (1964). Myoglobin free-radicals. *Biochim. Biophys. Acta* **88**, 235-236.
- Lewis, J. M., Grove, T. J. and O'Brien, K. M.** (2015). Energetic costs of protein synthesis do not differ between red-and white-blooded Antarctic notothenioid fishes. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **187**, 177-183.
- Macqueen, D. J. and Johnston, I. A.** (2014). Cardiac myoglobin deficit has evolved repeatedly in teleost fishes. *Biol. Lett.* **10**, 20140225.
- Meredith, M. P. and King, J. C.** (2005). Rapid climate change in the ocean west of the Antarctic Peninsula during the second half of the 20th century. *Geophys. Res. Lett.* **32**.
- Montgomery, J. and Clements, K.** (2000). Disaptation and recovery in the evolution of Antarctic fishes. *Trends Ecol. Evol.* **15**, 267-271.
- Moylan, T. J. and Sidell, B. D.** (2000). Concentrations of myoglobin and myoglobin mRNA in heart ventricles from Antarctic fishes. *J. Exp. Biol.* **203**, 1277-1286.
- Mueller, I., Hoffman, M., Dullen, K. and O'Brien, K.** (2014). Moderate elevations in temperature do not increase oxidative stress in oxidative muscles of Antarctic notothenioid fishes. *Polar Biol.* **37**, 311-320.
- Mueller, I. A., Devor, D. P., Grim, J. M., Beers, J. M., Crockett, E. L. and O'Brien, K. M.** (2012). Exposure to critical thermal maxima increases oxidative stress in hearts of white- but not red-blooded Antarctic notothenioid fishes. *J. Exp. Biol.* **215**, 3655-3664.
- Place, S. P. and Hofmann, G. E.** (2005). Constitutive expression of a stress-inducible heat shock protein gene, hsp70, in phylogenetically distant Antarctic fish. *Polar Biol.* **28**, 261-267.

- Place, S. P., Zippay, M. L. and Hofmann, G. E.** (2004). Constitutive roles for inducible genes: evidence for the alteration in expression of the inducible hsp70 gene in Antarctic notothenioid fishes. *Am. J. Physiol-Reg. I* **287**, R429-R436.
- Podrabsky, J. E. and Somero, G. N.** (2006). Inducible heat tolerance in Antarctic notothenioid fishes. *Polar Biol.* **30**, 39-43.
- Reeder, B. J. and Wilson, M. T.** (2005). Hemoglobin and myoglobin associated oxidative stress: from molecular mechanisms to disease states. *Curr. Med. Chem.* **12**, 2741-2751.
- Reinheckel, T., Ullrich, O., Sitte, N. and Grune, T.** (2000). Differential impairment of 20S and 26S proteasome activities in human hematopoietic K562 cells during oxidative stress. *Arch. Biochem. Biophys.* **377**, 65-68.
- Riccio, A., Gogliettino, M., Palmieri, G., Balestrieri, M., Facchiano, A., Rossi, M., Palumbo, S., Monti, G. and Cocca, E.** (2015). A new APEH cluster with antioxidant functions in the antarctic hemoglobinless icefish *Chionodraco hamatus*. *PLoS One* **10**.
- Robinson, E. and Davison, W.** (2008). The Antarctic notothenioid fish *Pagothenia borchgrevinki* is thermally flexible: acclimation changes oxygen consumption. *Polar Biol.* **31**, 317-326.
- Ruud, J. T.** (1954). Vertebrates without erythrocytes and blood pigment. *Nature* **173**, 848-850.
- Sandersfeld, T., Davison, W., Lamare, M., Knust, R. and Richter, C.** (2015). Elevated temperature causes metabolic trade-offs at the whole organism level in the Antarctic fish *Trematomus bernacchii*. *J. Exp. Biol.*, jeb. 122804.
- Seebacher, F., Davison, W., Lowe, C. J. and Franklin, C. E.** (2005). A falsification of the thermal specialization paradigm: compensation for elevated temperatures in Antarctic fishes. *Biol. Lett.* **1**, 151-154.
- Shang, F. and Taylor, A.** (2011). Ubiquitin–proteasome pathway and cellular responses to oxidative stress. *Free Radic. Biol. Med.* **51**, 5-16.
- Shang, F., Gong, X. and Taylor, A.** (1997). Activity of ubiquitin-dependent pathway in response to oxidative stress: ubiquitin-activating enzyme is transiently up-regulated. *J. Biol. Chem.* **272**, 23086-23093.

- Shang, F., Nowell, T. R. and Taylor, A.** (2001). Removal of oxidatively damaged proteins from lens cells by the ubiquitin-proteasome pathway. *Exp. Eye Res.* **73**, 229-238.
- Shibatani, T. and Ward, W. F.** (1995). Sodium dodecyl sulfate (SDS) activation of the 20S proteasome in rat liver. *Arch. Biochem. Biophys.* **321**, 160-166.
- Shringarpure, R., Grune, T., Mehlhase, J. and Davies, K. J.** (2003). Ubiquitin conjugation is not required for the degradation of oxidized proteins by proteasome. *J. Biol. Chem.* **278**, 311-318.
- Sidell, B. D. and O'Brien, K. M.** (2006). When bad things happen to good fish: the loss of hemoglobin and myoglobin expression in Antarctic icefishes. *J. Exp. Biol.* **209**, 1791-1802.
- Sidell, B. D., Vayda, M. E., Small, D. J., Moylan, T. J., Londrville, R. L., Yuan, M. L., Rodnick, K. J., Eppley, Z. A. and Costello, L.** (1997). Variable expression of myoglobin among the hemoglobinless Antarctic icefishes. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 3420-3424.
- Small, D. J., Vayda, M. E. and Sidell, B. D.** (1998). A novel vertebrate myoglobin gene containing three A+T-rich introns is conserved among Antarctic teleost species which differ in myoglobin expression. *J. Mol. Evol.* **47**, 156-166.
- Small, D. J., Moylan, T., Vayda, M. E. and Sidell, B. D.** (2003). The myoglobin gene of the Antarctic icefish, *Chaenocephalus aceratus*, contains a duplicated TATAAAA sequence that interferes with transcription. *J. Exp. Biol.* **206**, 131-139.
- Tappel, A. d.** (1955). Unsaturated lipid oxidation catalyzed by hematin compounds. *J. Biol. Chem.* **217**, 721-734.
- Todgham, A. E., Hoaglund, E. A. and Hofmann, G. E.** (2007). Is cold the new hot? Elevated ubiquitin-conjugated protein levels in tissues of Antarctic fish as evidence for cold-denaturation of proteins in vivo. *J. Comp. Physiol. B* **177**, 857-866.
- Turner, J., Colwell, S. R., Marshall, G. J., Lachlan-Cope, T. A., Carleton, A. M., Jones, P. D., Lagun, V., Reid, P. A. and Iagovkina, S.** (2005). Antarctic climate change during the last 50 years. *Int. J. Climatol.* **25**, 279-294.

- Vaughan, D. G., Marshall, G. J., Connolley, W. M., Parkinson, C., Mulvaney, R., Hodgson, D. A., King, J. C., Pudsey, C. J. and Turner, J.** (2003). Recent rapid regional climate warming on the Antarctic Peninsula. *Climatic Change* **60**, 243-274.
- Wickner, S., Maurizi, M. R. and Gottesman, S.** (1999). Posttranslational quality control: folding, refolding, and degrading proteins. *Science* **286**, 1888-1893.
- Windisch, H. S., Frickenhaus, S., John, U., Knust, R., Pörtner, H. O. and Lucassen, M.** (2014). Stress response or beneficial temperature acclimation: transcriptomic signatures in Antarctic fish (*Pachycara brachycephalum*). *Mol. Ecol.* **23**, 3469-3482.
- Witas, H., Gabryelak, T. and Matkovics, B.** (1984). Comparative studies on superoxide dismutase and catalase activities in livers of fish and other Antarctic vertebrates. *Comp. Biochem. Physiol. C Pharmacol. Toxicol.* **77**, 409-411.

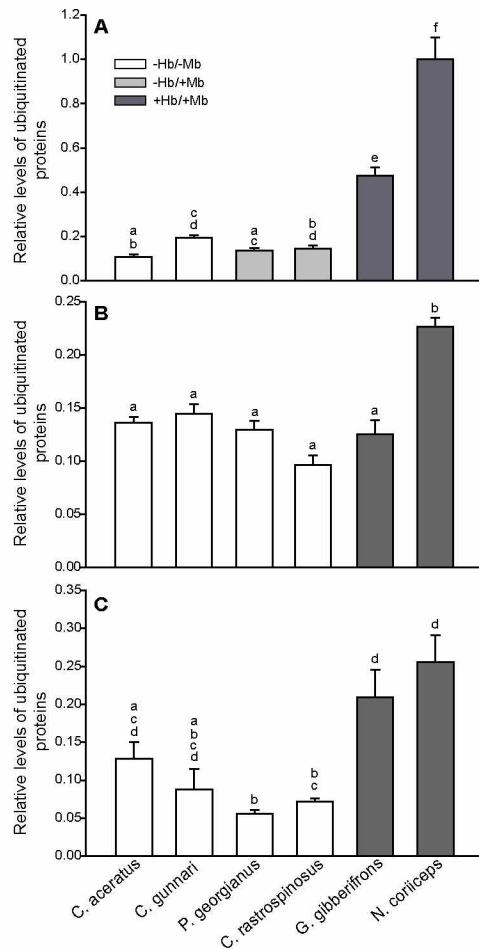


Figure 1.1 Levels of ubiquitinated proteins in tissues of notothenioids that vary in the expression of hemoglobin (Hb) and myoglobin (Mb). Relative levels of ubiquitinated proteins in heart ventricle (A), pectoral adductor muscle (B), and liver (C) of six species of notothenioids (all notothenioids lack Mb in pectoral adductor muscle and liver). Levels of ubiquitinated proteins were normalized to levels in heart ventricle of *N. coriiceps*. Values are means \pm s.e.m. (N=6). Significant differences among species and within each tissue are indicated by different letters ($P < 0.0083$ for A & C, $P < 0.05$ for B).

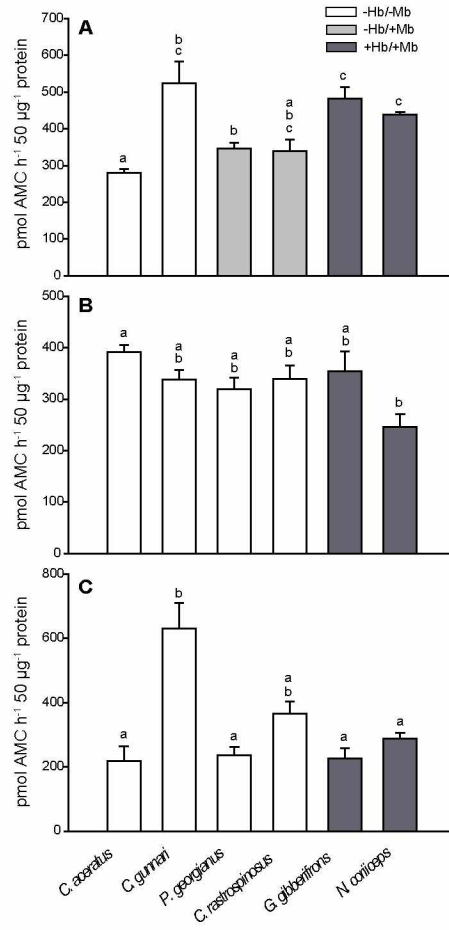


Figure 1.2 Activity of the 20S proteasome in tissues of notothenioids that vary in the expression of hemoglobin (Hb) and myoglobin (Mb). Activity of the 20S proteasome in heart ventricle (A), pectoral adductor muscle (B), and liver (C) of six species of notothenioids (all notothenioids lack Mb in pectoral adductor muscle and liver). Values are means \pm s.e.m. (N=8). Significant differences among species and within each tissue are indicated by different letters ($P < 0.0083$).

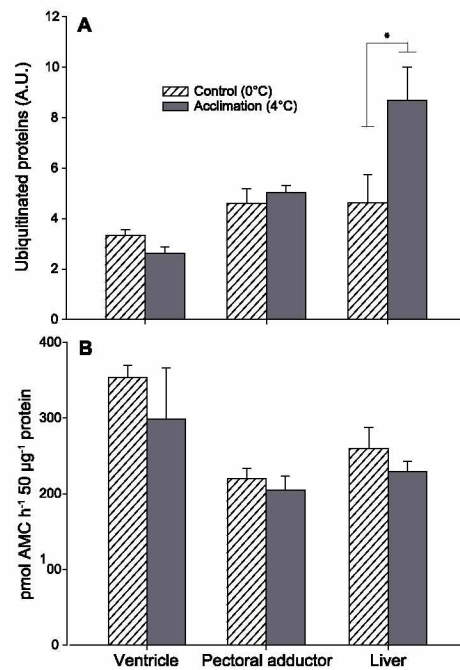


Figure 1.3 The effect of warm acclimation on protein ubiquitination and activity of the 20S proteasome. Relative levels of ubiquitinated proteins (A) and 20S proteasome activity (B) in *N. coriiceps* acclimated to 4°C and held at ambient temperature (0° C) in heart ventricle (N = 5-6), pectoral adductor muscle (N = 7 for each treatment), and liver (N = 5-6). Values are means \pm s.e.m. Significant differences are denoted by an asterisk. (P < 0.05).

General Conclusions

The results from my study indicate that icefishes (family Channichthyidae) have lower levels of oxidized and ubiquitinated proteins but they do not degrade proteins at a higher rate compared to red-blooded notothenioids. Levels of ubiquitinated proteins were higher in all three tissues of the red-blooded *Notothenia coriiceps* than in icefishes, but 20S proteasome activity was not. This suggests that although red-blooded notothenioids have higher levels of ubiquitinated proteins, they are not degrading these proteins at a higher rate. Further, there was no change in levels of ubiquitinated proteins in heart ventricle or pectoral adductor muscle, nor was there a change in 20S proteasome activity in any of the three tissues in response to acclimation to 4°C. These results suggest that the red-blooded notothenioid, *N. coriiceps*, has a limited ability to compensate to a 4°C temperature increase with respect to the ubiquitin-proteasome pathway (UPP).

Although I found that the rates of protein degradation by the 20S proteasome were not higher in notothenioids expressing hemoglobin (Hb) and myoglobin (Mb) compared to species lacking these proteins, other pathways of protein degradation may be more prominent in notothenioids. Recent studies suggest that a novel isoform of the enzyme acylpeptide hydrolase (APEH-2) is thermally stable at cold temperature and exhibits protease activity toward oxidized proteins *in vitro* (Gogliettino et al., 2014). Further, the proteolytic activity and relative expression of APEH-2 is higher in the red-blooded notothenioid *Trematomus bernacchii* compared to the icefish *Chionodraco rastrispinosus*, lending credence to the hypothesis that Hb and Mb contribute to protein oxidation and increased rates of protein degradation (Riccio et al., 2015). A series of experiments could be conducted to determine whether red-blooded notothenioids have higher rates of protein degradation through APEH-2 compared to icefishes. First, relative transcript levels of APEH-2 could be measured in notothenioids differing in Hb and Mb expression. Next, the proteolytic activity of APEH-2 could be measured in these same individuals, according to the methods used by Riccio et al. (2015). Measurements of indicators of oxidized proteins, such as levels of protein carbonyls and ubiquitinated proteins, should also be collected, as well as measurements of levels of Hb and Mb. Ideally, the measurements of each of these parameters would be acquired from multiple tissues (e.g. heart ventricle, pectoral adductor, liver, blood, spleen) in the same individual

fish, to identify correlating factors. In these experiments, a strong correlation between APEH-2 expression and activity, and levels of Hb and Mb would suggest that Hb and Mb contribute to protein oxidation.

My results suggest that the UPP is not altered in *N. coriiceps* after acclimation to 4°C for 22 days. Three weeks may not be a sufficiently long enough period of time for notothenioids to acclimate. Alternatively, a 4°C increase in temperature may not be sufficient to elicit a change in the UPP. It is also possible that warm acclimation doesn't alter rates of protein degradation. To distinguish among these possibilities, *N. coriiceps* could be acclimated to multiple temperatures (e.g. 4°C, 6°C, and 8°C) for up to 60 days (depending on the length of the field season) and levels of ubiquitinated proteins and 20S proteasome activity measured in the heart ventricle, pectoral adductor muscle, and liver of fishes acclimated for different lengths of time (e.g. 4 weeks, 6 weeks, and 8 weeks). This study design would allow us to determine whether 4°C was too small a temperature increase or 3 weeks was too short an acclimation period to alter levels of ubiquitinated proteins or 20S proteasome activity in *N. coriiceps*.

My data indicate that the loss of Hb and Mb does not impart an energetic advantage for icefishes, further supporting the consensus opinion that the loss of these proteins is the result of neutral mutations. This suggests that the cold, oxygen-rich environment of the Southern Ocean, in concert with a reduction in competition resulting from widespread extinctions of the fish fauna, permitted the survival of icefishes lacking oxygen-binding proteins. Overall, this research increases our understanding of the ability of notothenioids to acclimate to warmer water with respect to the UPP, indicating that a 4°C increase in temperature does not alter rates of protein degradation. This contrasts with a similar study in the temperate spotted wolffish (*Anarhichas minor*), which showed that rates of 20S proteasome activity increased with a 4°C increase in temperature (Lamarre et al., 2009). Ultimately, the inability of *N. coriiceps* to compensate for increased temperature could be an indication that the warming of the Western Antarctic Peninsula may adversely affect notothenioids.

References

- Bilyk, K. T. and Cheng, C. H. C.** (2014). RNA-seq analyses of cellular responses to elevated body temperature in the high Antarctic cryopelagic nototheniid fish *Pagothenia borchgrevinki*. *Mar. Genomics* **18, Part B**, 163-171.
- Clarke, A. and Johnston, I. A.** (1996). Evolution and adaptive radiation of Antarctic fishes. *Trends Ecol. Evol.* **11**, 212-218.
- Davies, K. J.** (2001). Degradation of oxidized proteins by the 20S proteasome. *Biochimie* **83**, 301-310.
- DeWitt, H. H. and Bushnell, V. C.** (1971). Coastal and deep-water benthic fishes of the Antarctic: American Geographical Society New York.
- Dröge, W.** (2002). Free radicals in the physiological control of cell function. *Physiol. Rev.* **82**, 47-95.
- Dudek, E., Shang, F., Valverde, P., Liu, Q., Hobbs, M. and Taylor, A.** (2005). Selectivity of the ubiquitin pathway for oxidatively modified proteins: relevance to protein precipitation diseases. *FASEB J.* **19**, 1707-1709.
- Eastman, J.** (2005). The nature of the diversity of Antarctic fishes. *Polar Biol.* **28**, 93-107.
- Eastman, J. T.** (1993). Antarctic Fish Biology: Evolution in a Unique Environment. San Diego: Academic Press.
- Enzor, L. A. and Place, S. P.** (2014). Is warmer better? Decreased oxidative damage in notothenioid fish after long-term acclimation to multiple stressors. *J. Exp. Biol.* **217**, 3301-3310.
- Gogliettino, M., Riccio, A., Balestrieri, M., Cocca, E., Facchiano, A., D'Arco, T. M., Tesoro, C., Rossi, M. and Palmieri, G.** (2014). A novel class of bifunctional acylpeptide hydrolases—potential role in the antioxidant defense systems of the Antarctic fish *Trematomus bernacchii*. *FEBS J.* **281**, 401-415.
- Grove, T. J., Hendrickson, J. W. and Sidell, B. D.** (2004). Two species of Antarctic icefishes (genus *Champsocephalus*) share a common genetic lesion leading to the loss of myoglobin expression. *Polar Biol.* **27**, 579-585.

- Hartley, A., Cooper, J. M. and Schapira, A. H. V.** (1993). Iron induced oxidative stress and mitochondrial dysfunction: Relevance to Parkinson's disease. *Brain Res.* **627**, 349-353.
- Hawkins, A. J. S.** (1991). Protein turnover: a functional appraisal. *Funct. Ecol.* **5**, 222-233.
- Honda, K., Casadesus, G., Petersen, R. B., Perry, G. and Smith, M. A.** (2004). Oxidative stress and redox-active iron in Alzheimer's disease. *Ann. N. Y. Acad. Sci.* **1012**, 179-182.
- Kennett, J. P.** (1977). Cenozoic evolution of Antarctic glaciation, the circum-Antarctic Ocean, and their impact on global paleoceanography. *J. Geophys. Res.* **82**, 3843-3860.
- King, J. and Harangozo, S.** (1998). Climate change in the western Antarctic Peninsula since 1945: observations and possible causes. *Ann. Glaciol.* **27**, 571-575.
- King, N. K., Looney, F. D. and Winfield, M. E.** (1964). Myoglobin free-radicals. *Biochim. Biophys. Acta* **88**, 235-236.
- Lamarre, S. G., Le Francois, N. R., Driedzic, W. R. and Blier, P. U.** (2009). Protein synthesis is lowered while 20S proteasome activity is maintained following acclimation to low temperature in juvenile spotted wolffish (*Anarhichas minor* Olafsen). *J. Exp. Biol.* **212**, 1294-1301.
- Lee, M. H., Hyun, D. H., Jenner, P. and Halliwell, B.** (2001). Effect of proteasome inhibition on cellular oxidative damage, antioxidant defences and nitric oxide production. *J. Neurochem.* **78**, 32-41.
- Littlepage, J. L.** (1965). Oceanographic investigations in McMurdo Sound, Antarctica. *Biology of the Antarctic Seas II.* **5**, 1-37.
- Meredith, M. P. and King, J. C.** (2005). Rapid climate change in the ocean west of the Antarctic Peninsula during the second half of the 20th century. *Geophys. Res. Lett.* **32**, L19604-L19608.
- Moylan, T. J. and Sidell, B. D.** (2000). Concentrations of myoglobin and myoglobin mRNA in heart ventricles from Antarctic fishes. *J. Exp. Biol.* **203**, 1277-1286.

- Mueller, I., Hoffman, M., Dullen, K. and O'Brien, K.** (2014). Moderate elevations in temperature do not increase oxidative stress in oxidative muscles of Antarctic notothenioid fishes. *Polar Biol.* **37**, 311-320.
- Reeder, B. J. and Wilson, M. T.** (2005). Hemoglobin and myoglobin associated oxidative stress: from molecular mechanisms to disease states. *Curr. Med. Chem.* **12**, 2741-2751.
- Riccio, A., Gogliettino, M., Palmieri, G., Balestrieri, M., Facchiano, A., Rossi, M., Palumbo, S., Monti, G. and Cocca, E.** (2015). A new APEH cluster with antioxidant functions in the Antarctic hemoglobinless icefish *Chionodraco hamatus*. *PLoS One* **10**.
- Ruud, J. T.** (1954). Vertebrates without erythrocytes and blood pigment. *Nature* **173**, 848-850.
- Shang, F., Gong, X. and Taylor, A.** (1997). Activity of ubiquitin-dependent pathway in response to oxidative stress: ubiquitin-activating enzyme is transiently up-regulated. *J. Biol. Chem.* **272**, 23086-23093.
- Sidell, B. D., Vayda, M. E., Small, D. J., Moylan, T. J., Londrville, R. L., Yuan, M. L., Rodnick, K. J., Eppley, Z. A. and Costello, L.** (1997). Variable expression of myoglobin among the hemoglobinless Antarctic icefishes. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 3420-3424.
- Sies, H.** (1997). Oxidative stress: oxidants and antioxidants. *Exp. Physiol.* **82**, 291-295.
- Somero, G. N. and DeVries, A. L.** (1967). Temperature tolerance of some Antarctic fishes. *Science* **156**, 257-258.
- Tappel, A. L.** (1953). The mechanism of the oxidation of unsaturated fatty acids catalyzed by hematin compounds. *Arch. Biochem. Biophys.* **44**, 378-395.
- Todgham, A. E., Hoaglund, E. A. and Hofmann, G. E.** (2007). Is cold the new hot? Elevated ubiquitin-conjugated protein levels in tissues of Antarctic fish as evidence for cold-denaturation of proteins in vivo. *J. Comp. Physiol. B* **177**, 857-866.
- Turner, J., Colwell, S. R., Marshall, G. J., Lachlan-Cope, T. A., Carleton, A. M., Jones, P. D., Lagun, V., Reid, P. A. and Iagovkina, S.** (2005). Antarctic climate change during the last 50 years. *Int. J. Climatol.* **25**, 279-294.

- Vaughan, D. G., Marshall, G. J., Connolley, W. M., Parkinson, C., Mulvaney, R., Hodgson, D. A., King, J. C., Pudsey, C. J. and Turner, J.** (2003). Recent rapid regional climate warming on the Antarctic Peninsula. *Climatic Change* **60**, 243-274.
- Wickner, S., Maurizi, M. R. and Gottesman, S.** (1999). Posttranslational quality control: folding, refolding, and degrading proteins. *Science* **286**, 1888-1893.
- Yin, J., Overpeck, J. T., Griffies, S. M., Hu, A., Russell, J. L. and Stouffer, R. J.** (2011). Different magnitudes of projected subsurface ocean warming around Greenland and Antarctica. *Nature Geoscience* **4**, 524-528.

Appendix



(907) 474-7800
(907) 474-5993 fax
fyiacuc@uaf.edu
www.uaf.edu/iacuc

Institutional Animal Care and Use Committee

909 N Koyukuk Dr. Suite 212, P.O. Box 757270, Fairbanks, Alaska 99775-7270

May 7, 2013

To: Kristin O'Brien, PhD
Principal Investigator

From: University of Alaska Fairbanks IACUC

Re: [247598-11] Collaborative research: Redox balance in Antarctic notothenioid fishes: Do icefishes have an advantage?

The IACUC reviewed and approved the Amendment/Modification to the protocol referenced above by Designated Member Review.

Received:	May 6, 2013
Approval Date:	May 7, 2013
Initial Approval Date:	June 13, 2011
Expiration Date:	June 12, 2014

This action is included on the May 16, 2013 IACUC Agenda.

PI responsibilities:

- *Acquire and maintain all necessary permits and permissions prior to beginning work on this protocol. Failure to obtain or maintain valid permits is considered a violation of an IACUC protocol and could result in revocation of IACUC approval.*
- *Ensure the protocol is up-to-date and submit modifications to the IACUC when necessary (see form 006 "Significant changes requiring IACUC review" in the IRBNet Forms and Templates)*
- *Inform research personnel that only activities described in the approved IACUC protocol can be performed. Ensure personnel have been appropriately trained to perform their duties.*
- *Be aware of status of other packages in IRBNet; this approval only applies to this package and the documents it contains; it does not imply approval for other revisions or renewals you may have submitted to the IACUC previously.*
- *Ensure animal research personnel are aware of the reporting procedures on the following page.*

(The following information is also available in a printable format in the IRBNet Forms and Templates)

HOW DO I REPORT CONCERNS ABOUT ANIMALS IN A UAF RESEARCH FACILITY?

- All "live" animal concerns related to care and use should be reported to the IACUC
- Email: fyiacuc@uaf.edu Phone: 474-7800
- Report form: www.uaf.edu/iacuc/report-concerns/
- IACUC Committee Members: www.uaf.edu/iacuc/iacuc-info/
- Additional information: www.uaf.edu/ori/responsible-conduct/research-misconduct/ and www.uaf.edu/ori/responsible-conduct/conflict-of-interest/

WHAT SHOULD I DO IF AN ACCIDENT OR INCIDENT OCCURS IN AN UAF ANIMAL FACILITY?

- **For all immediate human emergencies call 911** or UAF Dispatch at 474-7721 for less immediate emergencies.
- If you have **suffered an animal bite or other injury**, complete an "Accident/Incident Investigation form" (personal injury) form available at www.uaf.edu/safety/incidentreport-2012.pdf.
- If an accident such as a **chemical spill** occurs, contact the Environmental Health, Safety, and Risk Management (EHS&RM) Supervisor at 474-5617 or the Hazmat Coordinator at 474-7889.

WHO DO I CONTACT IF I FIND A DEAD, INJURED, OR DISTRESSED ANIMAL IN A UAF RESEARCH FACILITY?

- During regular business hours, immediately contact facility staff and/or Veterinary Services Staff at 474-7020.
- After hours or on weekends, immediately contact facility staff and/or Veterinary Services Staff using the contact numbers posted on the "Emergency Contact Information" in the facility or call UAF Dispatch at 474-7721.
- Contact the IACUC at 474-7800 or fyiacuc@uaf.edu if an "Emergency Contact Information" sign is NOT posted in the facility.
- Contact the IACUC if you are not satisfied with the response from Vet Services.

HOW DO I REPORT ANY CONCERNS REGARDING WORK HAZARDS OR ANY GENERAL UNSAFE CONDITIONS?

- Complete an "Unsafe Condition Reporting Program" form, available at the EHS&RM website: www.uaf.edu/safety/unsafe-condition/

WHERE CAN I OBTAIN GENERAL OCCUPATIONAL SAFETY INFORMATION?

- www.uaf.edu/iacuc/occupational-health/